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Ubiquitination and its influence in boar sperm physiology and cryopreservation☆

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Abstract

Recent reports document the potential use of the ubiquitin protein as an indicator of mammalian sperm quality or fertility, based on poor morphology, sperm count, and other cellular qualities. However, its influence on cellular physiologic mechanisms and boar sperm cryopreservation are unknown. The objective of this research was to determine the influence of boar sperm ubiquitination ($n = 12$ boars) on motility (using CASA), and flow cytometry and fluorescent probes (in parentheses) to evaluate mitochondrial activity (JC-1), plasma and acrosomal membrane integrity (PI and FITC-PNA), membrane fluidity (M540), and chromatin stability (TUNEL) for fresh and frozen–thawed samples. The effects of ubiquitination (determined flow cytometrically) on the ability of frozen–thawed boar sperm to capacitate (FLUO-3AM) and acrosome react (FITC-PNA) were also investigated using flow cytometry. Cryopreservation induced a decrease in the percentage of sperm that were ubiquitinated from 29 to 20% ($P < 0.0001$), but no significant effects of ubiquitin on sperm quality (motility, membrane integrities and organization) were detected. The ability of sperm to capacitate and acrosome react was influenced by ubiquitination. Samples with more ubiquitinated boar sperm were able to maintain plasma membrane integrity (PMI) better and have fewer live acrosome-reacted cells over 120 min of induced capacitation ($P < 0.05$). In conclusion, frozen–thawed ubiquitinated boar sperm were better able to survive the physical stresses of induced capacitation, yet were still capable of capacitating and acrosome reacting, which may enable use of this assay for in the vitro evaluation of the quality of boar sperm.
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1. Introduction

Ubiquitination is an apoptotic mechanism in which defective cells are labeled by inserting a small protein (ubiquitin) into the plasma membrane. Sperm are reportedly ubiquitinated in order to remove imperfect cells by the proteasome in the testis [1] and by phagocytosis in the epididymis [2]. Therefore, higher numbers of fertile sperm are present in the ejaculate than if this mechanism was not in place. Several sperm quality measurements have been analyzed and correlated (positively or negatively) with ubiquitination [3–8]. In addition, the amount of ubiquitinated sperm in a semen sample has been correlated with infertility or poor quality (motility, morphology, etc.) in humans [3,7], stallions [4], bulls [2], and boars [5,8].

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Interpretation of the effects of sperm ubiquitination on physiologic characteristics was limited in these analyses, as only single time-point evaluations of morphology, motility, concentration, count, and other basic characteristics were conducted, either immediately following ejaculation, or immediately following cryopreservation, rather than over a time course. Furthermore, sperm ubiquitination may alter other physiologic processes than those investigated. Because ubiquitination of sperm is the addition of a protein to the plasma membrane, greater levels could influence membrane fluidity, and cryopreservation success, as these are influenced by the protein, cholesterol, and phospholipid contents of the plasma membrane [9]. Therefore, the addition of ubiquitin to the sperm membrane could modulate sperm quality, membrane physiology, and ultimately affect the cryosurvival of sperm.

The objectives were to determine: (1) if ubiquitination influences sperm quality (motility, mitochondrial function, and membrane integrities) prior to and following cryopreservation; (2) if ubiquitination affects the membrane organization (membrane fluidity); and (3) if ubiquitination affects frozen–thawed sperm physiology (capacitation and the acrosome reaction evaluated over time). If ubiquitination exerts an influence on sperm physiology, ultimately these analyses could be used as a tool for in vitro evaluation of sperm quality.

2. Materials and methods

2.1. Experimental design

Boar semen was collected and analyzed for three experiments during the month of April. In Experiment 1, ubiquitination of sperm was determined using single-parameter (fresh and frozen–thawed sperm analyzed only for the percentage of ubiquitinated sperm) and dual-parameter (frozen–thawed sperm analyzed simultaneously for ubiquitination and TUNEL/chromatin damage) flow cytometry. Ubiquitin data (single- and dual-parameter flow cytometric analyses) acquired in Experiment 1 were analyzed to compare techniques, to determine if both methods provided similar results. Also in Experiment 1, the effects of ubiquitination of boar sperm on fresh and frozen–thawed motility using computer automated semen analysis, plasma membrane integrity (PMI), acrosomal membrane integrity, and mitochondrial function using flow cytometry, were also analyzed. Results from both the single- and dual-parameter analyses were then used for analytical purposes in Experiments 2 and 3. Experiment 2, an investigation of membrane dynamics, analyzed the influence of ubiquitination on fresh and frozen–thawed sperm plasma membrane fluidity using flow cytometry. Finally, in Experiment 3, frozen–thawed boar sperm were analyzed using flow cytometry for capacitation status (intracellular calcium), acrosomal membrane status, and PMI over 120 min of incubation to induce capacitation, and to determine if there was a relationship between ubiquitination and these physiological processes.

2.2. Collection, processing, and cryopreservation of boar semen

Semen was collected from boars (18–24 months of age) using the gloved-hand technique and the gel-free fraction was separated using sterile gauze. The boars ($n = 12$; one ejaculate per boar used for all subsequent analyses) were from composite lines (Line 1, $n = 6$; Line 2, $n = 5$; and Line 3, $n = 1$), housed in the same facility, fed a diet that met 100% of their nutritional needs, and given water ad libitum. Each semen sample was diluted 1:4 (v:v) with 37 °C Androhep Plus (295 mOsm; Minitube of America, Verona, WI, USA), cooled to 23 °C over 1 h, and then cooled to 15 °C over an additional 1.5 h. Samples were placed in insulated boxes that maintained the temperature at 15 °C, and shipped to the laboratory overnight.

Upon arrival, samples were centrifuged at $800 \times g$ for 10 min at 15 °C and the supernatant removed. The sperm in the resulting pellet, prior to cryopreservation, will be referred to as the fresh sample for the remainder of the analyses. Sperm concentration was determined using a spectrophotometer specifically calibrated for boar sperm [10].

Boar sperm samples were diluted using BF5 cooling extender (CE; 52 mM TES, 16.5 mM Tris [hydroxymethyl] aminomethane, 178 mM glucose, 20% egg yolk; 325 mOsm) in two-thirds of the final volume, and cooled to 5 °C over 2.5 h [11]. Samples were then diluted with BF5 freezing extender (91.5% CE, 6% glycerol, 2.5% Equex Paste, Minitube of America, v:v; one-third of the final volume; 1450 mOsm) [11] so that the final sperm concentration was 200×10^6 sperm/mL and loaded into 0.5-mL CBS straws (IMV Corporation, Minneapolis, MN, USA). The samples were frozen in liquid nitrogen vapor using a Minidigitcool UJ400 programmable freezer (IMV Corporation) with the following freeze rate; 5 to −8 °C at −20 °C/min; −8 to −120 °C at −69 °C/min; −120 to −140 °C at −20 °C/min. The straws were then plunged into liquid nitrogen

for storage. Samples were thawed by submerging a semen straw in a 50 °C water bath and gently agitating the straw for 20 s. Thereafter, the straws were maintained at 37 °C and processed immediately for analysis, as described in the following sections.

2.3. Flow cytometry

Analysis was performed using a CyAn-ADP flow cytometer (Dako-Cytomation, Fort Collins, CO, USA). The flow cytometer was equipped with a 488-nm argon laser at 150 mW of power. Each analysis included a 95% reduction filter, a 545-nm dichroic long pass filter, a 640-nm dichroic long pass filter, a 730-nm dichroic long pass filter in the instance of Cy-5 use, and a 530/40-nm band pass filter to detect acrosomal membrane integrity using FITC-PNA (Sigma–Aldrich, St. Louis, MO, USA), mitochondrial function using JC-1 (Molecular Probes, Eugene, OR, USA), and intracellular calcium using Fluo-3 AM (Sigma–Aldrich) and a 613/20-nm band pass filter to detect sperm that were counter-stained with propidium iodide (PI; Molecular Probes) in each of the analyses. A 530/40-nm band pass filter to detect ubiquitin using FITC-labeled ubiquitin antibodies (Zymed Laboratories, S. San Francisco, CA, USA) was used when ubiquitin alone was analyzed. When ubiquitin and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) were analyzed simultaneously, a 530/40-nm band pass filter to detect TUNEL positive (Roche Diagnostics, Indianapolis, IN, USA) sperm and a 680/30-nm band pass filter to detect Cy-5 (Zymed Laboratories) positive/ubiquitinated sperm [2,3] were used. Membrane fluidity/capacitation status was analyzed using a 530/40-nm band pass filter to detect Yo-Pro-1 (Molecular Probes) fluorescence (plasma membrane integrity), and a 575/25-nm band pass filter to detect Merocyanine 540 (membrane fluidity/capacitation status; Molecular Probes). A minimum of 5000 sperm was analyzed from each sample using the flow cytometer. For analysis of the frozen–thawed samples, a single straw, from the original single ejaculates frozen per boar, was used for each experiment.

2.4. Experiment 1

2.4.1. Assessment of ubiquitin and TUNEL

Ubiquitin binding was determined using the assays described by Sutovsky et al. ([2,3]; bull and human sperm, respectively) and Kuster et al. ([5]; boar sperm). For single-parameter analysis (ubiquitin only), samples of fresh and frozen–thawed boar sperm were centrifuged at $800 \times g$ for 10 min and the supernatant was removed. Aliquots of the sperm pellets, 100×10^6 sperm, were fixed in 2% formaldehyde in phosphate buffered saline (PBS, Sigma–Aldrich; pH 7.4) for 40 min. The samples were centrifuged at $500 \times g$ for 5 min, blocked in 5% normal goat serum (NGS, Sigma–Aldrich) in PBS for 25 min, and incubated with the MK-12-3 anti-ubiquitin monoclonal antibody (MBL, Nagoya, Japan) at a 1:100 dilution. The samples were washed three times ($500 \times g$ for 5 min) with 1 mL of PBS containing 1% NGS and incubated with FITC-goat anti-mouse IgG (H + L) (Zymed Laboratories) diluted 1:80 in 1% NGS. Finally, the samples were washed twice with 1.5 mL of serum-free PBS ($500 \times g$ for 5 min) and suspended in 0.5 mL of serum-free PBS. Negative controls of each boar sperm sample were prepared by performing the preparation as stated, except the primary antibody incubation was omitted. Ubiquitinated sperm were determined by subtracting the negative control population from the population that fluoresced in samples treated with both antibodies, using the subtraction function of the CyAn-ADP flow cytometry software [3].

Simultaneous ubiquitin/TUNEL analysis (frozen–thawed samples) was performed according to the manufacturer's instructions in the In Situ Cell Death Detection Kit, AP (Roche Diagnostics) [2]. Samples were initially prepared for TUNEL labeling and then stained for ubiquitin analysis as described previously. After fixation, samples were permeabilized with 0.1% Triton-X 100 (Sigma–Aldrich) for 10 min, and then treated with the TUNEL reaction mixture for 1 h [2]. Samples were then washed and treated with the anti-ubiquitin antibody, as described previously, except that the secondary antibody used for labeling ubiquitin positive sperm was conjugated with Cy-5 (Zymed Laboratories) to avoid fluorescence emission overlap [2].

2.4.2. Motility analyses

For both fresh and frozen–thawed sperm, motility was determined using computer automated semen analysis (CASA; Hamilton Thorne Motility Analyzer, Beverly, MA, USA). The CASA was set up as follows: 30 frames acquired; frame rate of 60 Hz; minimum contrast of 55; minimum cell size of 5 pixels; VAP cutoff of 20 μm; progressive minimum VAP cutoff of 45 μm/s; VSL cutoff of 5 μm/s; static head size of 0.53–4.45; and magnification of 1.89 (which were preset by the manufacturer). A minimum of seven fields and 1000 sperm were observed for motility analysis.

2.4.3. Membrane integrity analyses

Samples (1×10^6 cells) of fresh and frozen–thawed boar sperm were diluted in 0.5 mL of Beltsville Thawing Solution (BTS; 205 mM glucose, 20.4 mM sodium citrate, 14.9 mM sodium bicarbonate, 3.4 mM EDTA, and 10 mM potassium chloride; 300 mOsm) [11] and stained with FITC-PNA (10 μL of a 1 mg/mL solution in water) and PI (5 μL of a 2.4 mM solution in water) to determine acrosomal integrity (FITC-PNA) and plasma membrane integrity (PI), respectively, of pre-freeze and frozen–thawed samples. The analysis was based on the technique created with boar sperm of Flesch et al. [12]. Samples were stained at 23 °C for 10 min prior to flow cytometric analysis for fresh samples.

2.4.4. Mitochondrial activity

Mitochondrial function was evaluated in fresh and frozen–thawed samples according to Garner et al. [13], which was adapted for boar sperm by Huo et al. [14]. Stock solutions of JC-1 (1.53 mM in Me_2SO) and PI, as described previously, were prepared. Boar sperm, 3×10^6 cells, was stained with 28 μL of JC-1 (stock solution) and 5 μL of PI (stock solution) in 100 μL (final volume) of BTS, and incubated in the dark for 30 min at 37 °C. An aliquot of the stained sperm, 50 μL, was diluted in BTS to 1 mL and analyzed flow cytometrically. Data presented are the percentage of membrane intact sperm (PI negative) with high mitochondrial membrane potential (JC-1 positive) [13].

2.5. Experiment 2

2.5.1. Plasma membrane fluidity

Detection of plasma membrane fluidity of fresh and frozen–thawed boar sperm was performed using the fluorescent stains Merocyanine-540 and Yo-Pro-1, based on the technique created with boar sperm by Harrison et al. [15]. Stock solutions of Merocyanine-540 (5 mM in Me_2SO) and Yo-Pro-1 (1 mM in Me_2SO) were prepared and frozen in aliquots. Working solutions of the stains Merocyanine-540 (50 μM) and Yo-Pro-1 (100 μM) were prepared from the stock solutions using Tyrodes (97 mM NaCl, 31 mM KCl, 0.3 mM Na_2HPO_4, 25 mM $NaHCO_3$, 2 mM $CaCl{\cdot}2H_2O$, 0.4 mM $MgCl{\cdot}6H_2O$) [16] and Me_2SO, respectively. Samples (1×10^6 sperm in 1 mL BTS) were stained with 5 μL of the Merocyanine-540 and 1 μL of the Yo-Pro-1 working solutions at 23 °C for 10 min in dim lighting, and analyzed using flow cytometry. Data presented are the percentage of membrane intact sperm (Yo-Pro-1 negative) with low plasma membrane fluidity/highly ordered membranes (Merocyanine-540 positive). Two populations of Merocyanine-540 positive cells were typically observed; low fluorescence/low fluidity and highly ordered, and high fluorescence/high fluidity and greater disorder, which was associated with sperm which were capacitating and/or acrosome reacting [15].

2.6. Experiment 3

2.6.1. Induced capacitation and acrosome reaction analyses

Frozen–thawed boar sperm (20×10^6 per boar sperm sample) were diluted in separate test tubes to 1 mL (final volume) in 10 mM calcium TALP (97.4 mM sodium chloride, 3.1 mM potassium chloride, 0.15 mM sodium phosphate, 24.9 mM sodium bicarbonate, 10 mM calcium chloride, 0.4 mM magnesium chloride hexahydrate, 0.2 mM sodium pyruvate, 25.3 sodium lactate, 5 mM glucose, 10 mM Hepes, and 0.3% (w/v) bovine serum albumin; 300 mOsm) [17] and incubated at 39 °C. The samples were maintained at this temperature for the duration of this analysis. Aliquots (50 μL) were removed at 15-min increments from 0 to 120 min, and diluted in DPBS containing Fluo-3 AM (10 μL of Sigma–Aldrich prepared solution) and PI [18], or FITC-PNA and PI. The samples were stained for 10 min and analyzed for percentages of live sperm (PI negative) that were exhibiting high levels of intracellular calcium (Fluo-3AM positive) and in separate analyses for percentages of live acrosome-reacted sperm (FITC-PNA positive). In the calcium analyses, two populations of calcium positive sperm were observed, with greater fluorescence indicating greater amounts of intracellular calcium [19].

2.7. Statistics

All percentage data were arc-sine transformed. The ANOVA procedure of SAS [20] was used to determine differences in the percentages of ubiquitinated sperm for the main treatment effects of fresh single-parameter flow cytometry, frozen–thawed single-parameter flow cytometry, frozen–thawed dual-parameter flow cytometry, boar and line. Differences in the means were separated using the Student–Newman–Keuls test [20].

Fresh or frozen–thawed percentages of sperm from the motility, plasma membrane integrity, live acrosomal membrane integrity, and membrane fluidity, assays were analyzed for correlations (PROC CORR) with the percentage of fresh or frozen–thawed ubiquitin positive sperm (independent variables) in separate analyses [20].

The induced capacitation analyses contained a repeated measures aspect; therefore, the mixed procedure of SAS was used [20]. The model included the fixed effects of the percentage of frozen–thawed ubiquitinated sperm per sample, time, and their interactions. Each sample (one sample per boar) was treated as the block in the complete block design [21]. The dependent variables analyzed included sperm plasma membrane integrity, acrosomal integrity, and intracellular calcium, in separate analyses.

3. Results

There were differences in the percentage of ubiquitinated sperm for cryopreservation status (fresh; $29.4 \pm 11\%$ or frozen–thawed; $20 \pm 12.5\%$; $P < 0.0001$) when samples were analyzed using single-parameter flow cytometry. In addition, dual-parameter flow cytometry resulted in the identification of significantly fewer ubiquitinated sperm ($0.51 \pm 0.42\%$) than either the fresh or frozen–thawed ubiquitination determined from the single-parameter assays ($P < 0.0001$). Comparison of methods for determination of ubiquitination (Experiment 1) revealed that the fresh ($r = -.61$) and frozen–thawed percentage of ubiquitinated sperm ($r = -.65$) determined with single-parameter analysis were inversely correlated with sperm that were ubiquitinated and had chromatin damage (evaluated only on frozen–thawed sperm; UR; dual-parameter assay; Fig. 1), and with sperm that are positive for chromatin damage alone (UL, Fig. 1; fresh, $r = -.58$; and frozen–thawed, $r = -.61$; $P < 0.05$).

The percentage of fresh and frozen–thawed ubiquitinated sperm (single-parameter flow cytometry) was not correlated with the overall quality of boar sperm (motilities, plasma and acrosomal membrane integrities, and mitochondrial function; Experiment 1) or with membrane fluidity (Experiment 2; $P > 0.05$). When boar sperm were evaluated using dual-parameter flow cytometry, the UR + LR populations (% ubiquitinated sperm chromatin damaged or not) were correlated with frozen–thawed motility ($r = .72$; $P < 0.01$), frozen–thawed progressive motility ($r = .63$; $P < 0.05$), and frozen–thawed plasma membrane integrity ($r = .64$; $P < 0.05$). The TUNEL positive sperm (UL + UR; chromatin damaged ubiquitinated or not) were only correlated with the percentage of live acrosome reacted sperm that were present immediately after thawing ($r = .70$; $P = .01$).

In Experiment 3, ubiquitination did not affect the ability of boar sperm to incorporate calcium intracellularly when induced to capacitate over 120 min ($P > 0.05$). However, ubiquitination, determined from single-parameter flow cytometry, influenced the viability of boar sperm during induced capacitation analysis (Fig. 2); samples having greater percentages of ubiquitinated sperm maintained plasma membrane

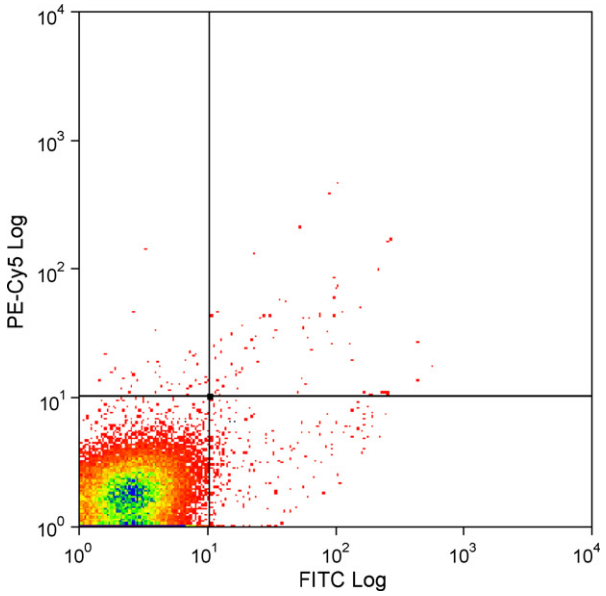

Fig. 1. Scatter plot of flow cytometric analysis of ubiquitinated boar sperm (FITC-Log) and TUNEL positive/chromatin damaged sperm (PE-Cy 5). Percentages of sperm were identified as unstained/no ubiquitination/chromatin intact (lower left; LL), ubiquitin positive (lower right; LR), TUNEL positive (upper left; UL), and ubiquitin positive/TUNEL positive (upper right; UR).

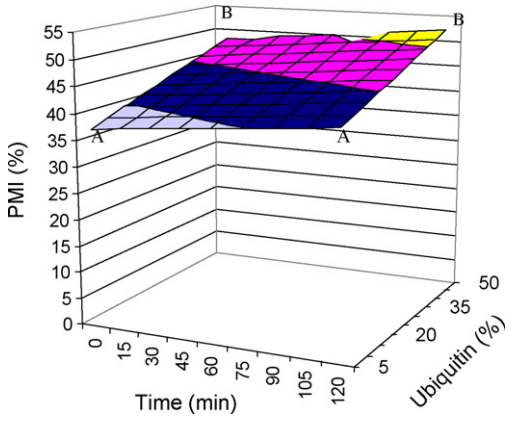

Fig. 2. The effect of time (0–120 min) and percent of sperm sample ubiquitinated on plasma membrane integrity (PMI; propidium iodide negative) for frozen–thawed boar sperm induced to capacitate and acrosome react ($P < 0.05$). As an example, a sample with a lower percentage of ubiquitinated sperm (A) will have significantly less plasma membrane intact sperm than that of a sample containing a greater percentage of ubiquitinated sperm (B), when evaluated over time in an induced capacitation/acrosome reaction analysis.

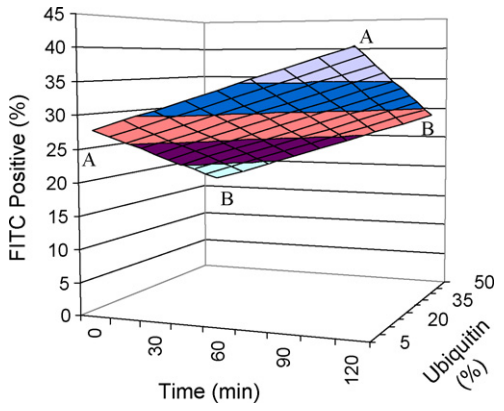

Fig. 3. The effect of time (0–120 min) and percent of sperm sample ubiquitinated on the percentage of plasma membrane intact (propidium iodide negative)/acrosome reacted frozen–thawed boar sperm that were induced to capacitate and acrosome react ($P < 0.05$). As an example, a sample with a lower percentage of ubiquitinated sperm (A) will have significantly more live acrosome reacted sperm than that of a sample containing a greater percentage of ubiquitinated sperm (B) when evaluated over time in an induced capacitation/acrosome reaction analysis.

integrity better than samples with fewer ubiquitinated sperm ($P < 0.05$). Furthermore, samples with greater percentages of ubiquitinated cells had fewer live acrosome-reacted sperm (Fig. 3) in comparison with samples containing fewer ubiquitinated sperm ($P < 0.05$).

4. Discussion

In numerous reports [1–5], semen samples containing large percentages of ubiquitinated sperm were indicative of a low quality sample and in some instances, this correlated negatively with fertility [1,3,4]. These findings were based on the concept that ubiquitination is a means for labeling defective sperm, which were lacking in chromatin integrity, and which should have been removed in the testis or epididymis [2]. Sutovsky et al. [2] found that sperm ubiquitination correlated with primary and total morphological abnormalities in bulls; furthermore, in boars, retained cytoplasmic and distal droplets were highly ubiquitinated [5]. Sutovsky et al. [4] also observed that the incidence of sperm ubiquitination increased in the stallion as the breeding season progressed from March (beginning of season and lowest percentage of ubiquitinated sperm) to December (end of season and highest incidence of ubiquitinated sperm). Initial observations also suggested that the sperm concentration and sperm count of stallions may be negatively correlated with the presence of ubiquitin [4]. Therefore, in a seasonal breeder, such as the stallion, ‘‘differential ubiquitination’’ may occur, thus regulating the spermatogenic cycle [4]. Further studies performed in a human fertility setting also indicated that ubiquitination correlated with differences in motility, count and morphology in males that were known to have male-factor infertility (high levels of ubiquitinated sperm) compared with males with proven fertility (low levels of ubiquitinated sperm) [7].

In addition, Lovercamp et al. [8] recently demonstrated the relationship of ubiquitination of boar sperm with fertility. The analyses, which were a modified version of the work of Sutovsky et al. [2], demonstrated that farrowing rate was correlated with the percentage of ubiquitinated sperm in a sample and, similar to Sutovsky et al. [4], changes in sperm ubiquitination were evident due to changes in the season.

Other research contradicted these findings, namely that by Muratori et al. [6], who demonstrated that sperm ubiquitination, was correlated with higher quality sperm (number, motility and morphology). When M540/ apoptotic bodies and other debris were removed from these samples (and consequently from the analyses), it became apparent that morphologically normal and viable sperm were labeled with ubiquitin [6]. Varum et al. [22] identified morphologically abnormal sperm that were not ubiquitinated and found no correlations with semen characteristics when the percentage of ubiquitinated sperm were analyzed. The current research supported the findings of Muratori et al. [6] and Varum et al. [22] which indicated that the presence of the ubiquitin protein was not necessarily indicative of a low quality sperm sample.

The question of utilizing the assay for estimating boar fertility or sample fertilizing potential is paramount. Predicting the fertility of a semen sample or individual male accurately depends on a number of factors related to sperm attributes, storage conditions, sample handling, as well as fertilization aspects and subsequent embryonic and fetal development [23]. To say that any one sperm laboratory assay will predict the fertility of a semen sample is questionable, but should more properly be stated as saying that evaluation of a sperm attribute may detect a sub-fertile semen sample because of the limiting scope of the evaluation [24]. Consequently, many studies are flawed because their conclusions about predicting fertility are based solely on one aspect of spermatozoa quality, function, or characteristics. By ignoring other sperm attributes and focusing solely on sperm ubiquitination, which appears to be the goal of the sperm tag immunoassay for humans

[7], many incorrect evaluations of semen, will be made because motility evaluation, membrane integrity, capacitation status and acrosomal membrane integrity analyses would not be performed. Furthermore, even in a study with a small sample size, such as this, the negative influence of ubiquitination on sperm physiology and cryobiology is not readily apparent as other authors have purported [1–5]. Thus, ubiquitin or ubiquitin/TUNEL analyses, if determined to be useful, must be utilized in conjunction with other analyses, so that multiple aspects of sperm quality are evaluated and a truer understanding of the viability of the sample is ascertained.

In order to utilize the ubiquitin analysis methodology, it is necessary to compare the methods of determination of sperm ubiquitination, the single- and dual-parameter flow cytometric analyses, and evaluate the legitimacy of the assays. Comparison of the results of the fresh and post-thaw ubiquitination analyses revealed significant differences among all three evaluations. It is important to emphasize what is being evaluated and how the samples are being analyzed; namely fresh single-parameter, frozen–thawed single-parameter, or frozen–thawed dual-parameter flow cytometric analysis. The decrease in the percentage of ubiquitinated sperm from fresh to frozen–thawed was understandable, as cryopreservation induced plasma membrane protein loss as well as plasma membrane protein and lipid rearrangement [9,25], although these results contradicted other findings [7]. However, the significant differences in detected ubiquitin between the frozen–thawed single- and dual-parameter analyses were not understandable, as the results should have been identical as both assays were detecting sperm ubiquitination. This raises concerns about this methodology; in particular the processing of the sperm for dual-parameter analysis, as this is the difference between the two frozen–thawed analyses. For the dual-parameter analysis, the samples were fixed and permeabilized so that the TUNEL staining could be achieved. In theory, the fragile frozen–thawed sperm could be damaged by the processing, so that the cells were de-ubiquitinated, and the consequential lower ubiquitin levels were detected. However, this seems highly improbable, as the fixation and permeabilization methods are quite commonly used with sperm and other cell types. The processing for staining could be causing damage to the chromatin, thus resulting in false positive TUNEL-labeled/chromatin-damaged sperm. As a result, the findings in this research indicating that sperm quality indicators (motilities, plasma membrane integrity, and acrosomal membrane integrity) were correlated with results from the dual-parameter assay must be qualified, because a clear reason as to why the methods provide different results is not known. Furthermore, the low number of sperm identified using the dual-parameter assay makes the results suspect, as one would not expect less than 1% of sperm to determine the fertility of a sample. Therefore, if a method is to be employed for evaluating sperm quality, the single-parameter analysis is the better choice.

If analysis was only limited to data from the single-parameter flow cytometric analyses, an influence of plasma membrane ubiquitination on boar sperm physiology could be demonstrated. There were no significant correlations of ubiquitination with general sperm quality characteristics (Experiment 1) or membrane component alterations (Experiment 2), which may be attributed to the small sample size and large standard deviations. The capacitation analyses demonstrated that samples with higher percentages of ubiquitinated sperm maintained plasma membrane integrity and acrosomal membrane integrity better during induced capacitation and acrosome reactions. Perhaps ubiquitinated sperm were better suited to survive the stresses associated with cryopreservation and or induced post-thaw capacitation, possibly because of the greater amount of protein contained in or bound to the plasma membrane [25], resulting in a more correct structure. Consequently, the observed slower rates of capacitation and acrosome reactions in capacitation induced frozen–thawed sperm (Fig. 3) that were associated with ubiquitinated sperm may be beneficial, because of the greater longevity of these samples (Fig. 2). Frozen–thawed sperm have an even more limited lifespan compared with fresh sperm after insemination; therefore, if ubiquitinated sperm can survive longer, this may result in greater fertility potential.

However, because the samples were frozen–thawed sperm, and this was an induced capacitation/acrosome reaction in vitro test, the exact opposite may be true. Based on Fig. 3, perhaps sperm samples with lower percentages of ubiquitinated sperm were better suited to capacitating within an appropriate interval following freezing and thawing. In other words, although the presence of the ubiquitin protein was not substantial enough to alter cryopreservation success or membrane fluidity, it may be capable of modulating the capacitation and acrosome reaction processes of sperm, whether at the membrane dynamic level or by means of intracellular signaling. This may contribute to differences in fertility due to a decreased/slower rate of capacitation and acrosome

reactions, resulting in decreased fertility because the sperm are not prepared at the necessary time for fertilization. Furthermore, while the presence of ubiquitin influenced survivability (Fig. 2), that is to say, that greater percentages of ubiquitination were related to greater survivability during capacitation and the acrosome reaction, live sperm will be useless if they are incapable of capacitating and acrosome reacting.

In conclusion, the present findings demonstrated that ubiquitination may play a role in sperm physiology and fertility, but further understanding of its mechanism must be ascertained. Furthermore, because of the varied findings that correlate ubiquitin with sperm quality criteria, this analysis would be best utilized within a series of analyses to assess overall sample quality. Consequently, basic criterion such as motility and morphology, are not overlooked and false results (sperm with abnormal morphology that are not ubiquitinated) are not obtained. In addition, when sperm samples are analyzed for the percentage of ubiquitinated cells, it is highly recommended that the single-parameter flow cytometry method is used, both due to the simplicity and lack of confounding results with both fresh and frozen–thawed boar sperm.

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